Note

Electrotransformation of *Bacillus mojavensis* with fluorescent protein markers

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ABSTRACT

Gram-positive endophytic bacteria are difficult to transform. To study the interactions between *Bacillus mojavensis* and maize, a method was developed to transform the microbe by electroporation with three integration plasmids expressing green, cyan and yellow fluorescent proteins (GFP variants). GFP Transformations were verified by antibiotic selection, microscopy and amylase deficiency, based on incorporation of the plasmids through recombination with the amylase gene. Phenotypic expressions of endophytism and fungal antagonisms of the transformants were the same as wild type stains. This represents the first successful transformation of this endophytic species with GFP markers.

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The process of transforming Gram-positive bacteria with foreign DNA has seen beneficial technological advances in more recent times. While the transformation of Gram-negative bacteria like *Escherichia coli*, owing to the wealth of knowledge and ease of transformation protocols, is more common place, many laboratories struggle with incorporating foreign DNA into Gram-positive bacteria such as agriculturally important strains of *Bacillus*. Some of the earliest *Bacillus* transformation protocols delivered DNA by protoplasting (Anagnostopolous and Spizizen, 1961; Chang and Cohen, 1979), and transduction (Marrero et al., 1984), but these methods can be laborious and do not always yield the most efficient results. Over the past 2 decades several new techniques have been introduced that attempt to simplify methods of *Bacillus* transformation. Many of these newer procedures involve electroporation protocols to enhance the uptake of exogenous plasmids and DNA vectors of interest.

Some of the earliest *Bacillus* electroporation techniques were developed in the late 1980s (Belliveau and Trevors, 1989; Bone and Ellar, 1989; Lereclus et al., 1989) and produced significantly higher transformation efficiencies in comparison with the older transformation techniques. Since then, efforts in improving transformation protocols have showed progress in further increasing efficiency by making changes to electroporation buffers and amending electroporation parameters such as the amounts of voltage and capacitance (Silo-suh et al., 1994; Xue et al., 1999).

Continued studies on Gram-positive transformation are needed due to the importance of *Bacillus* and other Gram-positive bacteria to

medicine and agriculture among other uses. The use of Gram-positive bacteria as a biocontrol agent in agriculture is a relatively new endeavor that shows advantageous potential. Gram-positive bacteria are generally safer than Gram-negative bacteria, and the spore-forming character of *Bacillus* species lends a longevity advantage to its use as an agricultural agent. Hence the ease of manipulating and studying genetic material in *Bacillus* species will provide a better understanding of these microbes and uncover new ways in which they can be exploited for agriculture, medicine and bioremediation. However, the caveat for these Gram-positive bacteria is the difficulty in achieving any degree of transformation.

Bacillus mojavensis, a relatively new *Bacillus* species, was isolated from maize kernels and determined to be an endophytic strain (Bacon and Hinton, 2002). The bacterium was also determined to have biocontrol potential due to its inhibition of the mycotoxic and plant pathogenic fungus *Fusarium verticillioides*, and to induce a marked stimulation of growth and development in maize plants (Bacon and Hinton, 2002). In an effort to understand the relationship of this bacterium as an endophyte of maize, a transformation protocol was developed for *B. mojavensis*, which was modified from previous Gram-positive transformation procedures.

The bacteria used for transformation consisted of six strains of *B. mojavensis* (Tables 1 and 2). The fungus used to test for antagonism by wild type and transformed *B. mojavensis* strains was *Fusarium verticillioides* RRC408, a pathogenic strain of maize. For transformation the integration plasmids pSG1154 (green, *gfp*), pSG1192 (cyan, *cfp*), and pSG1193 (yellow, *yfp*) were used. These GFP cloning vectors were designed for the generation of C-terminal fluorescent protein fusion that integrate into the chromosomal by double homologous crossover at the *amyE* locus (Lewis and Marston, 1999).

Table 1
Transformation efficiency of integrative plasmids into *Bacillus mojavensis* RRC101

Plasmids	Genotype*	Transformation efficiency
pSG1154	*bla amyE3′ spc P^-_{xyl}gfp amyES′*	4.17×10^4 cfu/µg DNA
pSG1192	*bla amyE3′ spc P_{xyl}cfp amyES′*	3.25×10^4 cfu/µg DNA
pSG1193	*bla amyE3′ spc P_{xyl}yfp amyES′*	3.66×10^4 cfu/µg DNA

*The constructs and the plasmids used in this study originated from Feucht and Lewis (2001).

The method used for measuring antagonism was reported earlier (Bacon and Hinton, 2002). The test for endophytism was determined following the recovery of bacteria from surface disinfected 3-week-old maize plants (3140, Pioneer Hi-Breed International, Inc., Des Moines, IA) cultured in a sterile synthetic soil mix under growth room conditions (Bacon and Hinton, 2002). Disinfected plant leaves, stems and roots were plated on LB agar plates following the procedure described below for identifying positive colonies of transformants and presented in Fig. 1.

B. mojavensis RRC101 cells were rendered electrocompetent using the protocol of Xue et al. (1999) with some modifications. Five milliliters of Luria–Bertrani broth was inoculated with a single colony of *B. mojavensis* and incubated overnight at 30 °C with shaking at 250 rpm. On the following day, the overnight culture was diluted 10-fold in LB broth with 0.5 M sorbitol and incubated at 30 °C with shaking at 250 rpm until a value of 0.90 O.D. at 600 nm was attained. The culture was chilled on ice for 10 min, and then centrifuged at 5000 ×g for 5 min at 4 °C. The supernatant was decanted, and the pellet was resuspended in a solution of 0.5 M sorbitol, 0.5 M mannitol and 10% glycerol at half the diluted culture volume and centrifuged at 5000 g for 5 min at 4 °C. This step was repeated three times. The washed cells were resuspended in a sterile solution of 0.5 M sorbitol, 0.5 M mannitol and 10% glycerol at a 1:50 dilution, and dispensed in microcentrifuge tubes that were chilled at 4 °C.

A volume of 80 µl of the electrocompetent cells was pipetted into a chilled 1.5 ml tube and mixed with approximately 1 ug of plasmid. DNA and cells were transferred to a chilled 2 mm gap electroporation cuvette (Bio-Rad, Hercules, California) and incubated on ice for 5 min. The cells were then electroporated in a GenePulser Xcell electroporator (Bio-Rad) at a voltage of 1 kV, capacitance of 50 µF, resistance of 200 Ω and a time constant of 9 ms. The cells were then transferred to 1 ml of a LB broth containing 0.5 M sorbitol and 0.38 M mannitol and incubated at 30 °C for 2 h with shaking at 250 rpm. 50 µl of cell culture was then spread onto spectinomycin selective LB plates and incubated overnight at 30 °C. Successful *B. mojavensis* transformations survived the antibiotic selection medium, and underwent homologous recombination in the amylase gene. Transformants with integrated plasmids were first selected by overnight growth on LB agar amended with 65 µg/ml spectinomycin. Positive clones were then further verified with the amylase deficiency assay described below.

Each positive clone was transferred to two LB agar plates containing 1% starch; one plate was used for reference because clones typically do not survive the amylase deficiency assay.

Untransformed *B. mojavensis* was transferred to an LB agar plate with 1% starch as a negative control, and both plates were incubated overnight at 30 °C. A few iodine crystals were spread over an inverted Petri dish lid, and the sample or negative control plates were placed over the iodine crystals under a fume hood and exposed to the iodine vapors for 5 to 10 min. Positive colonies had blue stains, while negative colonies had a clear ring around the colony indicating hydrolysis of starch as shown in Fig. 1c.

The genes from all three transformants are under the control of the Pxyl promoter which required xylose induction. A volume of 3 to 5 ml of CH medium (Nicholson and Setlow, 1990) amended with 30 µg/ml spectinomycin and 1% xylose was inoculated along with the

transformed colonies that scored positive for both spectinomycin selection and amylase deficiency. The positive strains were then incubated overnight at 30 °C with shaking at 250 rpm. Cells were centrifuged at 5000 ×g for 1 min, and 3/4 of the supernatant was decanted and a drop of the transformed cells was placed onto a microscope slide. The cells were viewed under a Leica DM model 6000B fluorescent microscope at 100× with the appropriate filter sets at exposure times of approximately 1 s and photographed with a Hamamatsu digital camera, model ORCA-er. Cells that were successfully transformed with pSG1154 (*gfp*), pSG1192 (*cfp*) (Lewis and Marston, 1999) or pSG1193 (*yfp*) (Feucht and Lewis, 2001) expressed the corresponding fluorescent proteins, and the green and blue fluorescence are shown as detected by fluorescent microscopy (Fig. 2). Transformed cells expressing fluorescence from *gfp, cfp,* and *yfp* were viewed through filter sets 470/40 nm, 436/20 nm, and 500/20 nm, respectively.

Following the above procedure, *B. mojavensis* RRC101 was transformed with integrative plasmids that contain gene sequences for the green, cyan and yellow fluorescent protein markers (Lewis and Marston, 1999), which will be useful in future quantitation and localization studies in an effort to establish the extent of endophytic colonization by Gram-positive endophytic bacteria in plant tissue. These plasmids were designed specifically for *Bacillus* species with transformation under traditional conditions (Anagnostopolous and Spizizen, 1961), which proved to be entirely unsuitable for *B. mojavensis.* Transformation efficiencies using variations of those methods resulted in no transformations. Using our procedure, the electrotransformation of *B. mojavensis* RRC101 results in an average transformation efficiencies for the three plasmids of 3.69×10^4. This is similar to, although somewhat less than transformations achieved for related species of *Bacillus* (Belliveau and Trevors, 1989; Lewis and Marston, 1999; Xue et al., 1999). The procedure described herein represents optimal conditions and proved critical for the transformation process of this difficult to transform species. This procedure was also successfully used to transfer the followings strains of *B. mojavensis*: ATCC 5151 isolated from the Mojave Desert; ATCC 51517 isolated from the Gobi Desert; NRRL B-14714 isolated from the Sahara Desert; NRRL B-14814 isolated from the Arizona desert; and RRC112 and RRC111, mutants of RRC101 that was isolated from maize (Table 2). The successful transformation of these different geographically different and distinct strains (Bacon et al., 2006), and mutants

Table 2
Endophytic characterization and antagonism to *Fusarium verticillioides* RRC408 from *Bacillus mojavensis* wild type strains and their transformed variants (T) with pSG1154 (green)

	Endophytic trait[a]	Antagonism zone (cm)[b]
101 (Corn kernels, Italy)	Yes	11.50a
101T	Yes	10.20a
112 (transformed mutant of 101)	Yes	11.80a
112T	Yes	10.64a
ATCC 51516 (Mohave)	Yes	7.12a
ATCC 51516T	Yes	6.49a
ATCC 51517 (Gobi)	Yes	11.23a
ATCC 51517T	Yes	10.40a
B-14714 (Sahara)	Yes	9.10a
B-14714T	Yes	8.89a
B-14814 (Arizona)	Yes	11.20a
B-14814T	Yes	11.81a

[a] Strains were characterized by being endophytic upon recovery from 24-day-old surface disinfected maize plants grown under plant growth room conditions (Bacon and Hinton, 2002).
[b] Method of determining antagonism was based on measuring the zone of inhibition to *F. verticillioides* produced when *B. mojavensis* was co-cultured on nutrient agar for 9 days at 25 °C (Bacon and Hinton, 2002). Each analysis was replicated four times, and the result is an average of two experiments; within each couplet of wild type and its transformed strain (T), values followed by the same letter are not significantly different (paired *t*-test, P=0.05).
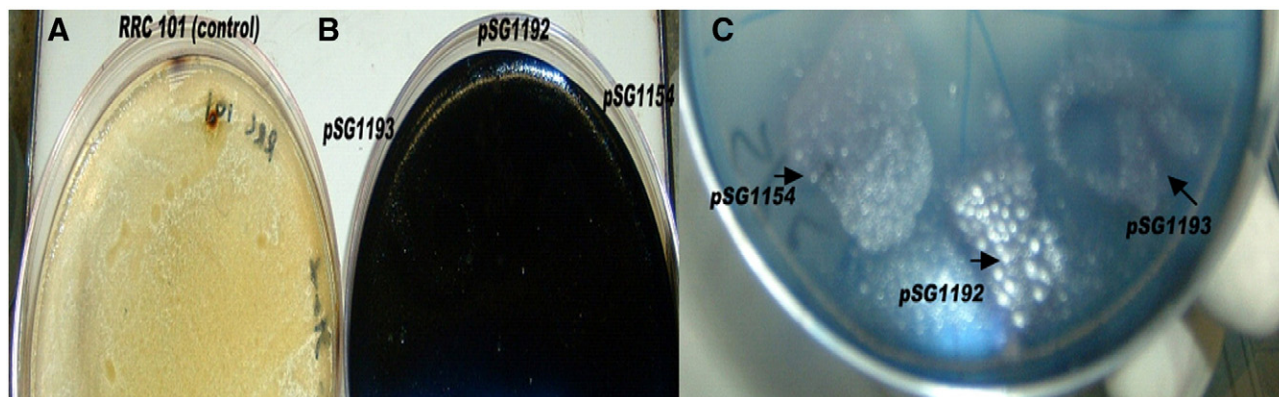
Fig. 1. *Bacillus mojavensis* strain RRC101 transformed with integrative plasmids pSG1154, pSG1192, pSG1193, by electroporation and subjected to the amylase test after spectinomycin selection. A) Control plate; B) *B. mojavensis* transformed with the 3 integrative plasmids; C) the same plate in B, 2 min into the assay.

demonstrates this method's suitability for transformation of strains within this endophytic species complex, and should prove suitable for other strains of this species recently assigned (Wang et al., 2007). This method also illustrates the utility of the procedure with several GFP plasmids. However, these plasmids are relatively similar and are designed for the generation of C-terminal fluorescent protein fusions that integrate into the chromosome by double homologous crossover at the *amyE* locus (Lewis and Marston, 1999). Further, similarity among plasmids that can infect *Bacillus* species is commonly known (Zawadzki et al., 1996).

The GFP transformants resembled the wild type strain RRC101 in being endophytic and antagonistic to *F. verticillioides* (Table 2), two essential attributes required for biocontrol uses. All strains transformed with pSG1154 (*gfp*) were endophytic and remained antagonistic to *F. verticillioides*. Further, the transformed strain, RRC101T, was identical to the wild type RRC101 in the utilization and growth characteristics on 92 substrates (data not reported) that was described earlier for the species wild type strains (Bacon et al., 2004). The substrate utilization and growth characteristics of the other transformed strains were not done, nor were phenotypic variations tested for the other strains transformed with pSG1192 (*cfp*) and pSG1193 (*yfp*). Nevertheless, the data comparing wild type strains transformed with pSG1154 indicate similar phenotypic traits, which suggest that other phenotypic traits may be equally unaltered. This is the first report of *B. mojavensis* transformation with GFP genes, which should facilitate the use of this endophytic species for delivery of desirable genes for the surrogate transformation of food crops.

Acknowledgements

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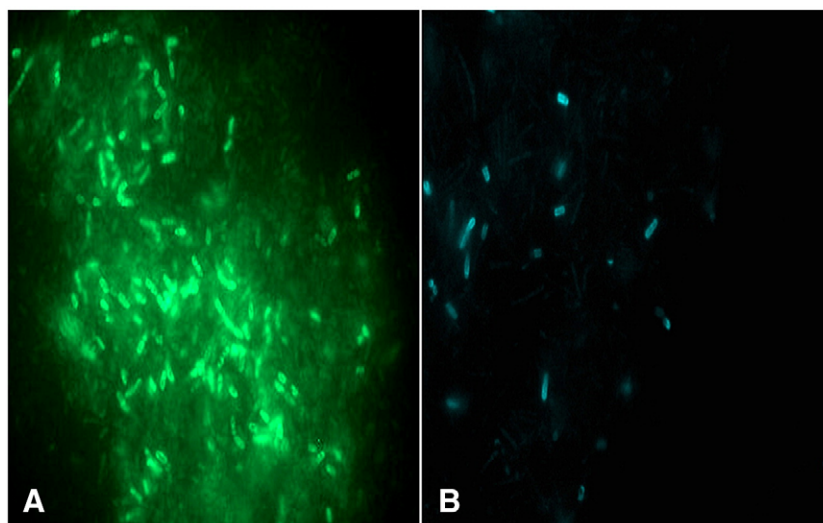

Fig. 2. *Bacillus mojavensis* transformed with A) pSG1154 (GFP) and B) pSG1192 (CFP), both viewed under a Leica DM 6000 fluorescent microscope at 100×.

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